CONTROL OF THE CIRCADIAN RHYTHM IN SEROTONIN CONTENT OF THE RAT PINEAL GLAND

BY SOLOMON H. SNYDER, MARK ZWEIG,* JULIUS AXELROD, AND JOSEF E. FISCHER

LABORATORY OF CLINICAL SCIENCE, NATIONAL INSTITUTE OF MENTAL HEALTH, NATIONAL INSTITUTES OF HEALTH

Communicated by Seymour S. Kety, December 3, 1964

Circadian rhythms have been demonstrated in mammals for a variety of biological phenomena, including running activity,[1] rectal temperature,[2] plasma eosinophil and corticosterone levels,[2, 3] liver mitoses, and nucleic acid content.[4] Certain of these rhythms are considered endogenous since they persist in the absence of environmental lighting.[1, 5] Controlling centers within the organism for these rhythms have been sought without success. Thus adrenal-dependent rhythms in epidermal mitoses and rectal temperature persist after hypophysectomy, although their amplitude is diminished.[6, 7]

In the rat pineal gland, marked circadian rhythms have been observed in serotonin[8] and melatonin[9] content and in the activity of the melatonin-forming enzyme.[10] This study was undertaken to examine possible mechanisms controlling the circadian rhythm in pineal serotonin content.

Methods.—Sprague-Dawley female rats (150–180 gm) were maintained under diurnal lighting conditions in clear plastic cages at a constant temperature of 25°C. An overhead fluorescent lamp provided about 110–150ft-c of illumination at the level of the cages. Unless otherwise noted, lights were kept on from 5 A.M. to 7 P.M. daily. Rats were killed by neck fracture at 1 P.M. and 11 P.M. In experiments involving constant light or dark environments, rats were kept in an isolated, soundproofed and air-conditioned room equipped with double-door light baffles. Pineal glands were removed immediately, placed on paper towels impregnated with cold isotonic saline, and weighed on a 25-mg Roller-Smith balance.

Single pineal glands were used for serotonin assay. After weighing, the pineal glands were homogenized in 0.5 ml of water with a tapered nylon rod in a 15-ml glass-stoppered centrifuge tube and frozen. Serotonin assays were performed on the following day by a modification of the ninhydrin procedure of Vanable[11] which is described elsewhere.[12] In this procedure serotonin was extracted from pineal gland homogenates, reacted with ninhydrin, and the resultant compound was measured fluorometrically.

Complete bilateral orbital enucleation was carried out under light ether anesthesia. Bilateral superior cervical ganglionectomy was performed under ether anesthesia. Superior cervical ganglia were decentralized in rats under ether anesthesia by removing a 1-cm segment of cervical sympathetic chain beginning 3 mm below the ganglion, with care to avoid disturbing the ganglion itself. Adrenalectomized, thyroidectomized, hypophysectomized, and oöphorectomized Sprague-Dawley female rats (150–180 gm) with matched control rats were obtained from Hormone Assay Laboratories.

Results.—The effect of varying lighting conditions and blinding on the circadian rhythm in serotonin content of the rat pineal gland: Groups of rats kept under diurnal lighting conditions were killed at 1 P.M. (6 hr of light) and 11 P.M. (4 hr of darkness), and their pineal glands were assayed for serotonin content. Serotonin levels were 2 to 3 times higher in pineal glands of rats killed at 1 P.M. than in those of rats killed at 11 P.M. (Table 1). These results essentially confirm the findings of Quay.[9] Groups of rats which had been kept under diurnal lighting for 1 week were transferred to constant light or dark environments. After 5 days these animals were killed at 1 P.M. and 11 P.M. and their pineal glands examined for serotonin (Table

TABLE 1

EFFECT OF CONTINUOUS LIGHT OR DARKNESS ON THE CIRCADIAN RHYTHM IN SEROTONIN CONTENT OF THE PINEAL GLAND

Group	Pineal serotonin (mμg/mg ± S.E.M.)
Diurnal light	
1 P.M.	72 ± 5.2
11 P.M.	34 ± 3.1*
Continuous light	
1 P.M.	65 ± 6.4
11 P.M.	62 ± 7.2
Constant darkness	
1 P.M.	68 ± 1.3
11 P.M.	32 ± 2.8*

*Differ from 1 P.M. values $p < 0.001$.
Groups containing 10 rats were maintained in continuous light or dark environments or under diurnal light conditions. After 5 days, serotonin content of their pineal glands was measured.

1). The circadian changes in pineal serotonin levels were abolished in rats kept in constant light. At 11 P.M. the pineal serotonin levels of rats maintained in continuous light were elevated to the 1 P.M. values found in diurnal lighting. In contrast to the effect of continuous light exposure, the circadian rhythm of pineal serotonin persisted unchanged in rats kept in continuous darkness. These results indicate that this circadian rhythm can be maintained in the absence of environmental lighting but can be suppressed by continuous light exposure.

The effects of blinding on the serotonin rhythm in the pineal gland are shown in Table 2. Both eyes were removed from groups of rats which were then maintained under diurnal lighting conditions for a 1- or 2-week period. The animals were then killed at 1 P.M. and 11 P.M. and their pineal glands assayed for serotonin. The serotonin rhythm was still present in both groups of blinded rats. To rule out the possible influence of extraretinal photoreceptors, blinded rats were kept in constant light or dark environments for 1 week and their pineal glands assayed for serotonin content at 1 P.M. and 11 P.M. (Table 3). In contrast to the suppressive effect of constant light exposure in normal rats, the serotonin rhythm persisted in blinded rats kept in a constant light environment.

To establish the rapidity with which continuous light abolishes the serotonin rhythm in the pineal gland, rats were placed in diurnal lighting conditions for 5 days. On the 6th day, groups of control and blinded rats were transferred to a room in which illumination was extended an additional 4 hr to 11 P.M. Pineal glands were removed at 1 P.M. and 11 P.M. on the 6th day and examined for serotonin content (Table 4). There was no reduction at 11 P.M. in the serotonin levels of the pineals of rats which were exposed to additional illumination. Blinded rats showed a fall in pineal serotonin content at 11 P.M. whether or not the lights had been turned off at 7 P.M. These observations indicate that the circadian rhythm in pineal serotonin content can be extinguished by exposure to an additional 4 hr of

TABLE 2

EFFECT OF BLINDING ON THE CIRCADIAN RHYTHM IN PINEAL SEROTONIN CONTENT

Treatment	Pineal serotonin (mμg/mg ± S.E.M.)
None	
1 P.M.	63 ± 5.9
11 P.M.	16 ± 3.1*
Blinded (1 week)	
1 P.M.	55 ± 4.8
11 P.M.	20 ± 2.8*
Blinded (2 weeks)	
1 P.M.	64 ± 9.7
11 P.M.	23 ± 3.4*

*Differ from 1 P.M. values $p < 0.001$.
Pineal glands of groups of 10 rats in diurnal lighting were examined for serotonin content 1 or 2 weeks after blinding.

TABLE 3

EFFECT OF CONTINUOUS LIGHT OR DARKNESS ON THE CIRCADIAN RHYTHM IN PINEAL SEROTONIN OF BLINDED RATS

Group	Pineal serotonin (mμg/mg ± S.E.M.)
Continuous light	
1 P.M.	63 ± 7.4
11 P.M.	22 ± 2.5*
Continuous darkness	
1 P.M.	56 ± 6.9
11 P.M.	23 ± 2.2*

*Differ from 1 P.M. values $p < 0.001$.
After 4 days in diurnal lighting, groups of 10 rats were blinded and transferred to constant light or dark environments. Seven days later, their pineals were assayed for serotonin.

light. Furthermore, the suppressive effect of additional light exposure requires intact retinae.

Effect of the removal of endocrine glands on the circadian rhythm in the serotonin content of the rat pineal gland: Previous work[5] indicated that the circadian rhythm of eosinophil count requires intact adrenal glands. To examine for possible influences of endocrine organs on the circadian rhythm in pineal serotonin, adrenalectomized, thyroidectomized, oöphorectomized, and hypophysectomized rats were kept in diurnal lighting, killed at 1 P.M. and 11 P.M. 1 week after operation, and their pineal glands examined for serotonin content (Fig. 1). The removal of these glandular tissues had no effect on the circadian serotonin rhythm.

TABLE 4

EFFECT OF ONE ADDITIONAL PERIOD OF LIGHT ON THE CIRCADIAN RHYTHM IN PINEAL SEROTONIN CONTENT

Group	Pineal serotonin (mμg/mg ± S.E.M.)
Control	
1 P.M.	66 ± 7.5
11 P.M. (lights off)	23 ± 4.1*
11 P.M. (lights on)	59 ± 6.3
Blinded	
1 P.M.	61 ± 7.0
11 P.M. (lights off)	18 ± 2.6*
11 P.M. (lights on)	13 ± 2.8*

*Differ from 1 P.M. values $p < 0.001$.
After 5 days' exposure of rats to diurnal lighting, some groups were transferred at 9 A.M. to a room in which lights were not turned off at 7 P.M. and the remainder kept in diurnal lighting conditions (lights off at 7 P.M.). All groups, containing 8 to 10 rats, were killed on the day of transfer.

The role of the sympathetic nervous system in the control of the circadian rhythm of serotonin content in the pineal gland: The major, if not the sole, innervation of the rat pineal gland is derived from sympathetic fibers whose cell bodies are located in the superior cervical ganglia.[13] It has been shown in this laboratory that the activities of hydroxyindole-O-methyl transferase, the melatonin-synthesizing enzyme,[14] and 5-hydroxytryptophan decarboxylase[15] in the pineal gland are affected by its sympathetic innervation. The influence of the sympathetic nerves on the circadian rhythm of serotonin in the pineal gland was examined in the following experiments. Groups of rats whose superior cervical ganglia had been bilaterally extirpated were kept in diurnal lighting for 6 days after the operation. These rats were examined for pineal serotonin content in the usual manner at 1 P.M. and 11 P.M. (Table 5). The circadian rhythm in pineal serotonin was completely abolished in ganglionectomized rats. The 1 P.M. values were lowered and the 11 P.M. values were elevated. These results differ somewhat from the suppressive action of constant light exposure which resulted in an elevation of 11 P.M. values with no change in 1 P.M. levels. These observations would suggest that constant light exposure and ganglionectomy differ in the mechanisms of their abolition of the pineal circadian serotonin rhythm.

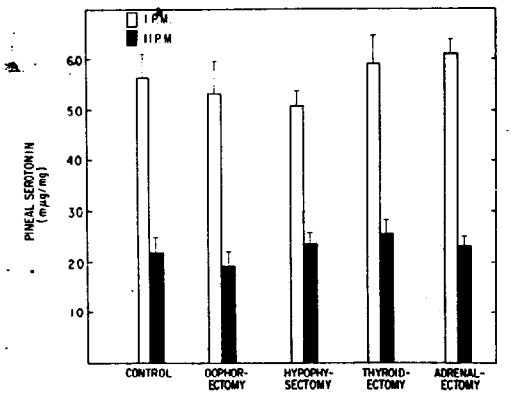

FIG. 1.—Lack of effect of removal of glands on the circadian serotonin rhythm in the rat pineal gland. Each group contained 10 rats. Vertical bars show the magnitude of the standard error of the mean.

There are several possible explanations for the suppressive action of superior cervical ganglionectomy: the circadian rhythm may be intrinsic to the pineal gland but requires intact sympathetic nerves for its expression; the controlling mechanism for the

TABLE 5

EFFECT OF SUPERIOR CERVICAL GANGLIONECTOMY ON THE CIRCADIAN RHYTHM IN RAT PINEAL SEROTONIN CONTENT

Treatment	Pineal serotonin (mμg/mg ± S.E.M.)
Sham-operated	
1 P.M.	70 ± 5.2
11 P.M.	27 ± 2.2*
Ganglionectomized	
1 P.M.	45 ± 2.1†
11 P.M.	42 ± 3.3†

* Differs from sham-operated 1 P.M. values $p < 0.001$.
† Differ from sham-operated 1 P.M. and 11 P.M. values $p < 0.001$.
Rats were kept in diurnal lighting for 6 days after operation and were killed on the 7th day. Each group contained 12–14 rats.

TABLE 6

EFFECT OF DECENTRALIZATION ON THE CIRCADIAN RHYTHM IN PINEAL SEROTONIN CONTENT

Group	Pineal serotonin (mμg/mg ± S.E.M.)
Sham-operated	
1 P.M.	66 ± 6.7
11 P.M.	18 ± 2.1*
Decentralized	
1 P.M.	42 ± 4.0†
11 P.M. (lights off)	44 ± 3.2†
11 P.M. (lights on)	43 ± 2.8†

* Differs from sham-operated 1 P.M. value $p < 0.001$.
† Differ from both sham-operated 1 P.M. and 11 P.M. values $p < 0.001$.
All rats were examined for pineal serotonin 6 days after operation. One group of decentralized rats was exposed to 4 hr of additional illumination before pineal gland removal.

rhythm could be localized in the superior cervical ganglia; the control might be neural but localized elsewhere in the body and communicated to the pineal gland via preganglionic fibers.

To examine this latter possibility, the preganglionic nerves to the superior cervical ganglia were severed bilaterally in groups of rats which were then maintained in diurnal lighting for 6 days before assaying their pineals for serotonin (Table 6). This treatment abolished the circadian rhythm in pineal serotonin in the same manner as did ganglionectomy. One group of decentralized rats was transferred, on the day they were killed, to a room in which the lights were not turned off at 7 P.M., and were killed at 11 P.M. Pineal serotonin levels for this group were the same as for decentralized groups under diurnal lighting. These results indicate that the serotonin rhythm is extrinsic to the pineal gland and communicated from the central nervous system to this organ via preganglionic sympathetic fibers to the superior cervical ganglia.

Discussion.—The circadian rhythm in serotonin content of the pineal gland is probably endogenous since it persists in the absence of environmental lighting and when other environmental cues (sound, temperature) are kept constant. Unlike endogenous circadian rhythms of eosinophil count, rectal temperature, plasma corticosterone levels, and running activity,[16] the endogenous serotonin rhythm is not influenced by the removal of the adrenal gland, nor is it affected by oöphorectomy, hypophysectomy, or thyroidectomy. Exposure to additional light for as little as 4 hr will completely suppress the rhythm by preventing the nocturnal decline in pineal serotonin content. In contrast to the almost immediate and complete abolition of the pineal serotonin rhythm by a single additional period of light, other endogenous rhythms, such as running activity in rodents,[1] are altered by continuous light exposure more gradually. As is the case with other circadian rhythms,[16] daily changes in light exposure might act as an external synchronizer (*Zeitgeber*) for the serotonin rhythm in the pineal gland.

Preliminary reports[17, 18] had shown that superior cervical ganglionectomy abolished the circadian rhythm in pineal serotonin content, although blinding was without effect.[17] These data, coupled with the present experiments on decentralization, clearly demonstrate that the controlling mechanism for the pineal serotonin

rhythm is extrinsic to this gland. Information regarding the control of the rhythm is probably transmitted from the central nervous system to the pineal gland via preganglionic fibers to the superior cervical ganglia where they synapse with postganglionic sympathetic nerves. Since the serotonin rhythm in the pineal gland, which is controlled by sympathetic centers in the central nervous system, closely resembles other endogenous circadian rhythms, it is possible that a similar central sympathetic mechanism may be involved in their control.

The sympathetic nervous system has also been implicated in the regulation of the circadian rhythm in melatonin synthesis in the pineal gland.[10] However, unlike the serotonin rhythm, the circadian variations in melatonin synthesis are not endogenous, but are directly influenced by external lighting information which is communicated to the pineal gland via the superior cervical ganglia.[10] Sympathetic nerves are necessary for other actions of light on the pineal gland. Constant light exposure elevates the activity of 5-hydroxytryptophan decarboxylase, the serotonin-forming enzyme, in the rat pineal gland and results in a decrease in pineal gland weight.[19, 20] Removal of the superior cervical ganglia abolishes the effect of light on this enzyme[15] and on pineal weight.[14]

Summary.—The presence of a circadian rhythm in serotonin content of the rat pineal gland has been confirmed. This rhythm persists in blinded rats and in rats kept in constant darkness but is abolished by one additional period of light. The suppressive effects of light exposure act via the retinae. The rhythm is unaffected by hypophysectomy, thyroidectomy, adrenalectomy, or oöphorectomy. Superior cervical ganglionectomy or decentralization of the superior cervical ganglia abolishes the pineal serotonin rhythm.

* NIH Special Fellow, first-year medical student, Washington University Medical School, St. Louis, Mo.

[1] Johnson, M. S., *J. Exptl. Zool.*, **82**, 315 (1939).

[2] Halberg, F., A. Zander, M. W. Houglum, and H. R. Mühlemann, *Am. J. Physiol.*, **177**, 361 (1954).

[3] Halberg, F., R. E. Peterson, and R. H. Silber, *Endocrinol.*, **64**, 222 (1959).

[4] Jardetzky, C. D., C. P. Barnum, and F. Halberg, *Am. J. Physiol.*, **187**, 608 (1956).

[5] Halberg, F., M. B. Visscher, and J. J. Bittner, *Am. J. Physiol.*, **174**, 109 (1953).

[6] Zander, H. A., J. Waerhaug, and F. Halberg, *J. Clin. Endocrinol. Metab.*, **14**, 829 (1954).

[7] Ferguson, D. J., M. B. Visscher, F. Halberg, and L. M. Levy, *Am. J. Physiol.*, **190**, 235 (1957).

[8] Quay, W. B., *Gen. Comp. Endocrinol.*, **1**, 3 (1963).

[9] Quay, W. B., *Proc. Soc. Exptl. Biol.*, **115**, 710 (1964).

[10] Axelrod, J., R. J. Wurtman, and S. H. Snyder, *J. Biol. Chem.*, in press.

[11] Vanable, J. W., *Anal. Biochem.*, **6**, 393 (1963).

[12] Snyder, S. H., J. Axelrod, and M. Zweig, *Biochem. Pharmacol.*, in press.

[13] Kappers, J. A., Jr., *Z. Zellforsch.*, **52**, 163 (1960).

[14] Wurtman, R. J., J. Axelrod, and J. Fischer, *Science*, **143**, 1328 (1964).

[15] Snyder, S. H., J. Axelrod, J. Fischer, and R. J. Wurtman, *Nature*, **203**, 4948 (1964).

[16] Halberg, F., E. Halberg, C. P. Barnum, and J. J. Bittner, in *Photoperiodism and Related Phenomena in Plants and Animals*, ed. R. B. Withrow (Washington: AAAS, 1959), p. 803.

[17] Snyder, S. H., M. Zweig, and J. Axelrod, *Life Sciences*, **3**, 1175 (1964).

[18] Fiske, V. M., *Science*, **146**, 253 (1964).

[19] Snyder, S. H., and J. Axelrod, *Federation Proc.*, **23**, 206 (1964).

[20] Fiske, V. M., K. Bryant, and J. Putnam, *Endocrinol.*, **66**, 489 (1960).